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(54) **NGR RECEPTOR AND METHODS OF IDENTIFYING TUMOR HOMING MOLECULES THAT HOME TO ANGIOGENIC VASCULATURE USING SAME**

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(57) **ABSTRACT**

The present invention provides a method of identifying a tumor homing molecule that homes to angiogenic vasculature by contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where the presence of specific binding identifies the molecule as a tumor homing molecule that homes to angiogenic vasculature. The invention also provides a method of directing a moiety to angiogenic vasculature in a subject by administering to the subject a conjugate including a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the moiety is directed to angiogenic vasculature. In addition, the invention provides a method of imaging the angiogenic vasculature of a tumor in a subject by administering to the subject a conjugate having a detectable moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor and detecting the conjugate.

3 Claims, 16 Drawing Sheets-

NGR RECEPTOR AND METHODS OF IDENTIFYING TUMOR HOMING MOLECULES THAT HOME TO ANGIOGENIC VASCULATURE USING SAME

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BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of cancer biology and drug delivery and, more specifically, to peptides that selectively home to a tumor, particularly a malignant tumor, to compositions comprising an agent such as a therapeutic agent conjugated to such tumor homing molecules, and to methods of using such molecules to target an agent to a tumor.

2. Background Information

Continuous developments over the past quarter century have resulted in substantial improvements in the ability of a physician to diagnose a cancer in a patient. For example, antibody based assays such as that for prostate specific antigen now allow early diagnosis of cancers such as prostate cancer. More recently, methods of genetic screening are becoming available to identify persons that may be particularly susceptible to developing a cancer. Genetic screening methods are based on the identification of one or more mutations in a gene that correlates with the development of a cancer. For example, the identification of genes such as BRCA1 and BRCA2 allowed the further identification of mutations in these genes that, in some cases, can correlate with susceptibility to developing breast cancer.

Unfortunately, methods for treating cancer have not kept pace with those for diagnosing the disease. Thus, while the death rate from various cancers has decreased due to the ability of a physician to detect the disease at an earlier stage, the ability to treat patients presenting with more advanced disease has advanced only minimally.

A major hurdle to advances in treating cancer is the relative lack of agents that can selectively target the cancer, while sparing normal tissue. For example, radiation therapy and surgery, which generally are localized treatments, can cause substantial damage to normal tissue in the treatment field, resulting in scarring and, in severe cases, loss of function of the normal tissue. Chemotherapy, in comparison, which generally is administered systemically, can cause substantial damage to organs such as bone marrow, mucosae, skin and the small intestine, which undergo rapid cell turnover and continuous cell division. As a result, undesirable side effects such as nausea, loss of hair and drop in blood cell count occur as a result of systemically treating a cancer patient with chemotherapeutic agents. Such undesirable side effects often limit the amount of a treatment that can be administered. Thus, cancer remains a leading cause of patient morbidity and death.

Efforts have been made to increase the target specificity of various drugs. For example, where a unique cell surface marker is expressed by a population of cells making up a tumor, an antibody can be raised against the unique marker and a drug can be linked to the antibody. Upon administration of the drug/antibody complex to the patient, the binding of the antibody to the marker results in the delivery of a relatively high concentration of the drug to the tumor.

Similar methods can be used where a particular cancer cell or the supporting cell or matrix expresses a unique cell surface receptor or a ligand for a particular receptor. In these cases, the drug can be linked to the specific ligand or to the receptor, respectively, thus providing a means to deliver a relatively high concentration of the drug to the tumor.

Tumors are characterized, in part, by a relatively high level of active angiogenesis, resulting in the continual formation of new blood vessels to support the growing tumor. Such angiogenic blood vessels are distinguishable from mature vasculature. One of the distinguishing features of angiogenic vasculature is that unique endothelial cell surface markers are expressed. Thus, the blood vessels in a tumor provide a potential target for directing a chemotherapeutic agent to the tumor, thereby reducing the likelihood that the agent will kill sensitive normal tissues. Furthermore, if agents that target the angiogenic blood vessels in a tumor can be identified, there is as likelihood that the agents can be useful against a variety of different types of tumors, since it is the target molecules in the angiogenic vessels that are recognized by such agents and not receptors specific for the tumor cells. However, the use of molecules that can bind specifically to tumor vasculature and target a chemotherapeutic agent to the tumor has not been demonstrated.

While linking a drug to a molecule that homes to a tumor can provide significant advantages for treatment over the use of a drug, alone, use of this method is severely limited by the scarcity of useful cell surface markers expressed in a tumor. Thus, a need exists to identify molecules that can selectively home to a tumor, particularly to the vasculature supporting the tumor. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a method of identifying a tumor homing molecule that homes to angiogenic vasculature of a tumor. The method includes the steps of contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where the presence of specific binding identifies the molecule as a tumor homing molecule that homes to angiogenic vasculature of a tumor. In a method of the invention, the substantially purified NCR receptor can be, for example, CD13/aminopeptidase N. If desired, the substantially purified NGR receptor can be immobilized on a support such as a plate or a bead.

The invention also provides a method of identifying a homing molecule that homes to angiogenic vasculature using substantially purified NGR receptor. The method includes the steps of contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where presence of specific binding identifies the molecule as a homing molecule that homes to angiogenic vasculature. The invention provides homing molecules that home to non-tumor angiogenic vasculature.

The present invention also provides a method of directing a moiety to angiogenic vasculature of a tumor in a subject by administering to the subject a conjugate including a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the moiety is directed to angiogenic vasculature of a tumor. In a method of the invention, the tumor homing molecule can be, for example, a peptide containing the sequence NGR, and, if desired, can be part of a conjugate in which the moiety is a cytotoxic agent, drug or cancer therapeutic agent, for example, doxo-

rubicin. A tumor homing peptide containing the sequence NGR can have, for example, the sequence CNGRCVSG-CAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or CNGRC (SEQ ID NO:8). In a method of the invention for directing a moiety to angiogenic vasculature of a tumor in a subject, the tumor homing molecule also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin and can be linked, if desired, to a drug moiety.

Further provided herein is a method of imaging the angiogenic vasculature of a tumor in a subject by administering to the subject a conjugate having a detectable moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the conjugate selectively binds the angiogenic vasculature, and detecting the conjugate. A detectable moiety for imaging angiogenic vasculature can be, for example, a radionuclide. A useful tumor homing molecule for imaging angiogenic vasculature can be, for example, a peptide containing the sequence NGR, such as a peptide containing the sequence CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or CNGRC (SEQ ID NO:8). A tumor homing molecule for imaging angiogenic vasculature also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl, fumagillin or another molecule that inhibits an aminopeptidase.

The invention also provides inhibitors of angiogenesis that are NGR receptor binding molecules. Such inhibitors can be, for example, an NGR receptor antibody or an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin, or a conjugate of such angiogenesis inhibitors to a drug or toxin.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows inhibition of in vivo phage homing by synthetic peptides. Recovery of phage displaying tumor homing peptides from breast carcinoma xenografts was measured after injection of phage or coinjection of the phage with various peptides. (A) Left panel: Recovery of phage expressing the NGR tumor homing peptide, CNGRCVSG-CAGRC (SEQ ID NO:3; "NGR phage") from tumor (filled bars) and brain (striped bars), and inhibition of the tumor homing by the soluble peptide CNGRC (SEQ ID NO:8). Middle panel: Recovery of CGSLVRC-phage and inhibition of the tumor homing by the soluble peptide CGSLVRC. Right panel: Recovery of RGD-4C phage (positive control; the peptide insert in the RGD-4C phage is CDCRGDCFC; SEQ ID NO:1) and unselected phage library mix (negative control). (B) Left panel: Increasing amounts of the RGD-4C soluble peptide were injected with the CNGRCVSGCAGRC-phage. Right panel: Increasing amounts of the CNGRC soluble peptide were injected with the RGD-4C phage.

FIG. **2** shows the specificity of tumor homing by the NGR phage relative to the positive control (RGD-4C) and negative control (fd-tet) phage.

FIGS. 3A to 3V show the immunohistochemical staining of the NGR phage in tumors and normal tissues following intravenous injection into nude mice bearing a human breast carcinoma or a human Kaposi's sarcoma. Samples were taken 4 min (FIGS. 3E, 3G, 3H and 3J) or 24 hr (FIGS. 3A to 3D, 3F, 3I, and 3K to 3V) after administration of the phage. FIGS. 3A, 3C, 3G and 3J are from mice receiving insertless phage (control phage) and FIGS. 3B, 3D, 3E, 3F, 3H, 3I and 3K to 3V are from mice receiving NGR phage. FIGS. 3A, 3B, 3E, 3F and 3G are breast tumor samples; FIGS. 3C, 3D, 3H, 3I and 3J are Kaposi's sarcoma samples; FIG. 3K is brain; FIG. 3L is lymph node; FIG. 3M is kidney; FIG. 3N is pancreas; FIG. 3O is uterus; FIG. 3P is mammary fat pad; FIG. 3Q is lung; FIG. 3R is intestine; FIG. 3S is skin; FIG. 3T is skeletal muscle; FIG. 3U is heart and FIG. 3V is urinary tract epithelium. Magnification: FIGS. 3A to 3D, 40×; FIGS. 3E to 3V, 200×.

FIG. **4** shows isolation of CD13/aminopeptidase N (APN) and CNGRC-phage binding to CD13/APN. (A) Aminopeptidase enzymatic activity in immuno-isolated CD13 detected by using the CD13 substrates Ala-pNA or Leu-pNA. (B) Phage binding to immuno-isolated CD13 from a Kaposi's sarcoma tumor cell octylglucoside extract. Phage carrying the indicated peptides were tested for binding to CD13 that was immobilized using an anti-CD13 antibody WM15 coated on microtiter wells. (C) Inhibition of NGR-phage binding to CD13 by an NGR-containing cyclic peptide (CNGRC; SEQ ID NO:8) but not by an RGD-containing cyclic peptide or by an unrelated peptide.

FIG. **5** shows binding of phage expressing an NGR containing peptide to cells transfected with CD13. CNGRC-phage binding to control Molt-4 cells and to Molt-4 cells transfected with CD13 and inhibition of the binding by the CNGRC cyclic peptide.

FIG. **6** shows affinity purification of CD13 from HL-60 cell extracts using an NGR peptide column and elution with CNGRC peptide. CD13 was detected in the fractions by using the WM15 monoclonal antibody, and normal mouse IgG was used as a negative control. Samples of each fraction were analyzed for aminopeptidase enzymatic activity by using Ala-PNA, and for matrix metalloprotease (MMP) activity by using an MMP substrate.

FIG. **7** shows upregulation of CD13 in cultured cells by angiogenic factors. Upregulation of CD13 by angiogenic factors in human umbilical cord endothelial cells (HUVECs).

FIG. **8** shows CD13-dependent cytotoxic activity of doxorubicin/CNGRC (CNGRC-dox) in vitro. (A) CD13-dependent cytotoxic activity of CNGRC-doxorubicin in vitro in activated HUVECs. (B) CD13-dependent activity of CNGRC-doxorubicin in vitro in CD13 transfected MDA-MB-435 breast carcinoma cells.

FIG. **9** shows recovery of CNGRC phage, RGD-4C phage, and control phage (insertless fd) from breast carcinoma xenografts, normal retina and angiogenic retina. (A) The experimental design is shown. (B) The ratio of recovered tetracycline-resistant to ampicillin-resistant phage is shown.

FIG. **10** shows the suppression of bFGF-induced angiogenesis in chicken chorioallantoic membrane and inhibition of tumor growth by CD13 antagonists. (A) CD13 antagonists, anti-CD13 antibody, bestatin and actinonin, suppress bFGF-induced angiogenesis. (B) Inhibition of the growth of 435 breast carcinoma tumors upon injection of a CD13 antagonist antibody.

FIG. **11** shows treatment of mice bearing MDA-MB-435-derived breast carcinomas and Hodgkin's lymphoma with doxorubicin-CNGRC peptide conjugate. Mice with size-matched tumors (~1000 mm^3) were randomized into four treatment groups (six animals per group): vehicle only, free doxorubicin (dox), doxorubicin-control peptide (dox-ctrl pep), and doxorubicin-CNGRC (dox-CNGRC). (A) Mice were treated at 5 μg/mouse/week of doxorubicin-equivalent. Difference in tumor volumes between day 1 and day 28 are shown. (B) A Kaplan-Meier survival curve of the mice in

Panel A is shown. (C) Mice bearing large (~5000 mm^3) MDA-MB-435 breast carcinomas (four animals per group) were randomized to receive a single-dose of free doxorubicin or doxorubicin-CNGRC conjugate at 200 μg/mouse of doxorubicin equivalent. A Kaplan-Meier survival curve is shown. (D) Mice bearing large (~5000 mm^3) Hodgkin's lymphoma (eight animals per group) were randomized to receive two doses of free doxorubicin plus unconjugated CNGRC peptide or doxorubicin-CNGRC conjugate at 40 μg/mouse of doxorubicin equivalent. A Kaplan-Meier survival curve is shown.

FIG. 12 shows the nucleotide and amino acid sequences of CD13/aminopeptidase N (SEQ ID NOS:200 and 201, respectively).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the identification of a target molecule responsible for the homing of molecules to angiogenic vasculature. The identified target molecule can act as a receptor, for example, for tumor homing molecules that home to the angiogenic vasculature of a tumor. As disclosed herein, various tumor homing molecules were isolated using in vivo panning; a core binding motif present in several of the tumor homing peptides was identified as the sequence NGR (see Example IV). In particular, phage expressing the peptides CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6) and CVLNGRMEC (SEQ ID NO:7) homed to human breast carcinomas, human Kaposi's sarcomas and mouse melanomas in mice bearing these tumors. Furthermore, such homing was competitively inhibited in vivo by the NGR containing peptide CNGRC (SEQ ID NO:8) but not by an unrelated peptide. Thus, the results disclosed herein indicate that a tumor homing peptide containing NGR can home to and specifically bind tumors of different types and species origin.

As further disclosed herein, a receptor that specifically binds tumor homing molecules containing the NGR motif has been identified (see Examples IX and X). Characterization of the NGR receptor revealed that this molecule immunoreacts with CD13 antibodies and that the isolated receptor specifically binds the NGR motif. Furthermore, the NGR receptor was expressed in angiogenic vasculature, including tumor vasculature, and is functionally important in angiogenesis. The identification of a target molecule such as the NGR receptor that is expressed in angiogenic vasculature can be advantageously used in vitro to identify new homing molecules, including high affinity ligands of the NGR receptor, as well as to target a tumor homing molecule and a linked moiety, such as a drug, to the angiogenic vasculature of a tumor in vivo.

Thus, the present invention provides a method of identifying a tumor homing molecule that homes to angiogenic vasculature of a tumor by using a substantially purified NGR receptor to identify the molecule. The method includes the steps of contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where the presence of specific binding identifies the molecule as a tumor homing molecule that homes to angiogenic vasculature of a tumor. A method of the invention directed to identifying a tumor homing molecule that homes to angiogenic vasculature of a tumor can additionally include the steps of administering an NGR binding molecule in vivo and determining binding of the NGR binding molecule to angiogenic vasculature. If desired, the substantially purified NGR receptor can be immobilized on a support such as a plate or a bead.

In a method of the invention, the substantially purified NGR receptor can be, for example, CD13/aminopeptidase N (FIG. 12; see, also, Look et al.,*J. Clin. Invest.* 83:1299–1307 (1989), which is incorporated herein by reference). This highly conserved transmembrane glycoprotein of about 150 kDa is incorporated into the cell membrane through an N-terminal hydrophobic segment (Look et al., supra, 1989; Xu et al., *Exp. Hematol.* 25:521–529 (1997), which is incorporated herein by reference). The large extracellular carboxy-terminal domain contains a pentapeptide that is characteristic of many zinc-dependent metalloproteases (Look et al., supra, 1989). Homologs of CD13 from several different species are well conserved (Look et al., supra, 1989; Xu et al., supra, 1997; Turner et al., in *Mammalian Ectoenzymes,* Kenny and Turner, eds., Elsevier Scientific Publishing Co., Amsterdam, p. 211 (1987)).

CD13 is expressed in normal and malignant cells of the myeloid lineage (Amoscato et al., *J. Immunol.* 142:1245–1252 (1989); Favaloro et al., *Br. J. Haematol.* 69:163–171 (1988); Makrynikola et al., *Exp. Hematol.* 23:1173–1179 (1995)) as well as in many epithelial, endothelial, and tumor cell types (Amoscato et al., *Biochem. Biophys. Acta* 1041:317–319 (1990); Rawlings and Barret, *Biochem. J.* 290:205–218 (1993); Mechtersheimer and Moller, *Am. J. Pathol.* 137:1215–1222 (1990); Menrad et al., *Cancer Res.* 53:1450–1455 (1993); Riemann et al., *J. Immunol.* 158:3425–3432 (1997)). CD13 can function differently depending on its location. In synaptic membranes, CD13 metabolizes enkephalins and endorphins (Matsas et al., *FEBS Lett.* 175:124–128 (1984)); in the intestinal brush border, it degrades regulatory peptides and scavenges amino acids (Turner et al., supra, 1997; Rawlings and Barret, supra, 1993); in lymphocytes, the cell surface activity of CD13 is associated with mitotic activation, antigen processing (Mouritsen et al., *J. Immunol.* 149:1987–1993 (1992); Falk et al., *Immunogenetics* 39:230–242 (1994)), cell adhesion, and migration (Menrad et al., supra, 1993; Saiki et al., *Int. J. Cancer* 54:137–143 (1993); Koch et al., *Am. J. Pathol.* 138:165–173 (1991)). In addition, CD13 has also been implicated in tumor invasion (Saiki et al., supra, 1993; Fujii et al., *Clin. Exp. Metastasis* 13:337–344 (1995)), signal transduction (O'Connell et al., *Transplant. Proc.* 21:3826–3827 (1989)), cell cycle control and differentiation (Makrynikola et al., supra, 1995;, Riemann et al., supra, 1997), and as a receptor for viruses (Delmas et al., *Nature* 357:417–420 (1992); Yeager et al., *Nature* 357:420–422 (1992)).

The expression levels and enzymatic activity of CD13 can be physiologically regulated, with the activity and substrate specificity of CD13 correlating with conformational changes and induced by various stimuli such as proliferative signals to cells. Studies using monoclonal antibodies also have indicated that CD13 undergoes regulatory intramolecular alterations that can result in the exposure of cryptic sites and can regulate enzyme activity. The presence of certain epitopes has also been related to prognosis of acute myeloid leukemia (Xu et al., supra, 1997; Favaloro et al., supra, 1988; Makrynikola et al., supra, 1995).

Cell-surface CD13/aminopeptidase N enzymatic activity can be potently blocked by bestatin, o-phenanthroline and actinonin (Taylor, *FASEB J.* 7:290–298 (1993); Rawlings and Barret, supra, 1993; Saiki et al., supra, 1993). Moreover, bestatin has been shown to possess immunomodulatory effects, and administration of high doses of bestatin results in marked suppression of experimental and spontaneous metastasis and inhibition of tumor cell invasion (Bruley-Rosset et al. *Immunol.* 38:75–83 (1979); van Hal et al., *J.*

Immunol. 153:2718–2728 (1994); Saiki et al., supra, 1993; Fujii et al., supra, 1995)). Although CD13 is expressed outside the vascular system, the results disclosed herein indicate that an NGR receptor having immunoreactivity with an anti-CD13 antibody is only exposed to the circulation in tumor vessels (see Example XII).

As used herein, the term "NGR receptor" means a target molecule that is expressed in angiogenic vasculature and that specifically binds an NGR motif. As described below and in Examples IX and X, an NGR receptor has been substantially purified and demonstrated to specifically bind several NGR containing peptides but not unrelated control peptides. The NGR receptor disclosed herein exhibits characteristics of a highly conserved transmembrane aminopeptidase designated CD13/aminopeptidase N (CD13/APN). As disclosed herein, an NGR receptor can be a transmembrane receptor. An NGR receptor also can be a molecule that immunoreacts with an anti-CD13 monoclonal antibody and that has aminopeptidase activity. An NGR receptor can have, for example, an amino acid sequence that is substantially similar to the amino acid sequence of CD13/APN (SEQ ID NO:201). Such an NGR receptor can have an amino acid sequence identical to the sequence of CD13/APN (SEQ ID NO:201) or can have one or more modifications, such as deletions, insertions or substitutions, including conservative and non-conservative amino acid substitutions, as long as the receptor remains expressed in angiogenic vasculature and retains specific NGR binding activity. The term "NGR receptor" also is intended to include polypeptides encompassing, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogues and mimetics, so long as such polypeptides retain functional activity as defined above.

A functional fragment of an NGR receptor also can be useful in the methods of the invention, for example, for identifying tumor homing molecules that home to angiogenic vasculature of a tumor. As used herein, the term "functional fragment," when used in reference to an NGR receptor, refers to a portion of an NGR receptor that retains some or all binding activity to a homing molecule. Such a functional fragment can be, for example, a domain that binds an NGR motif, such as the extracellular domain of an NGR receptor or an epitope specifically reactive with an antibody. A functional fragment of an NGR receptor useful in identifying a tumor homing molecule can be, for example, the extracellular carboxy-terminal domain of CD13/aminopeptidase N (Look et al., supra, 1989).

As used herein, the term "specific binding" means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity, for example, a peptide of similar size that lacks NGR. In this case, specific binding is indicated if the molecule has measurably higher affinity for the NGR receptor than the control molecule. Specificity of binding can be determined, for example, by competition with a control molecule that is known to bind to a target. For example, specific binding of an NGR peptide can be demonstrated by competing for binding with the same NGR peptide or a different peptide containing an NGR motif. In this case, specific binding is indicated if the binding of a molecule is competitively inhibited by the second NGR containing peptide.

The term "specific binding," as used herein, includes both low and high affinity specific binding. Specific binding can be exhibited, for example, by a low affinity homing molecule having a Kd of at least about 10^{-4} M. For example, if the receptor for a homing molecule has more than one binding site, a homing molecule having low affinity can be useful for targeting angiogenic vasculature. Specific binding also can be exhibited by a high affinity homing molecule, for example, a homing molecule having a Kd of at least about of 10^{-7} M, at least about 10^{-8} M, at least about 10^{-9} M, at least about 10^{-10} M, or can have a Kd of at least about 10^{-11} M or 10^{-12} M or greater. Both low and high affinity homing molecules are useful for targeting angiogenic vasculature.

The vasculature within a tumor generally undergoes active angiogenesis, resulting in the continual formation of new blood vessels to support the growing tumor. Such angiogenic blood vessels are distinguishable from mature vasculature in that angiogenic vasculature expresses unique endothelial cell surface markers, including the $\alpha_v\beta_3$ integrin (Brooks, *Cell* 79:1157–1164 (1994), which is incorporated herein by reference); WO 95/14714, Int. Filing Date Nov. 22, 1994) and receptors for angiogenic growth factors (Mustonen and Alitalo, *J. Cell Biol.* 129:895–898 (1995); Lappi, *Semin. Cancer Biol.* 6:279–288 (1995)). Moreover, tumor vasculature is histologically distinguishable from blood vessel in general in that tumor vasculature is fenestrated (Folkman, *Nature Med.* 1:27–31 (1995); Rak et al., *Anticancer Drugs* 6:3–18 (1995)). Thus, angiogenic vasculature is a particularly attractive target for targeting a tumor homing molecule. Such a tumor homing molecule can be useful for directing an agent such as a chemotherapeutic drug to a tumor, while reducing the likelihood the agent will have a toxic effect on normal, healthy organs or tissues (Examples VIII and XV). Moreover, a molecule that homes selectively to angiogenic vasculature also may have use in targeting other types of neovasculature such as that present in inflammatory, regenerating or wounded tissues. As used herein, the term "tumor homing molecule that homes to angiogenic vasculature of a tumor" means a molecule that can bind specifically to a target molecule expressed in angiogenic vasculature of a tumor. Similarly, the term "homing molecule that homes to angiogenic vasculature" means a molecule that can bind specifically to a target molecule expressed in angiogenic vasculature. It is understood that a homing molecule can be a tumor homing molecule.

A homing molecule can bind to angiogenic vasculature in a tumor or in non-tumor tissue. A homing molecule that binds to both tumor and non-tumor angiogenic vasculature also can exhibit preferential binding to tumor or non-tumor tissues. For example, a tumor homing peptide such as an NGR peptide can accumulate preferentially in angiogenic vasculature of tumors as compared to non-tumor angiogenic vasculature.

The invention also provides a method of identifying a homing molecule that homes to angiogenic vasculature using substantially purified NGR receptor. The method includes the steps of contacting a substantially purified NGR receptor with one or more molecules and determining specific binding of a molecule to the NGR receptor, where the presence of specific binding identifies the molecule as a homing molecule that homes to angiogenic vasculature.

A method of the invention for identifying a homing molecule also can include the steps of administering an NGR binding molecule in vivo and determining binding of the NGR binding molecule to angiogenic vasculature. Thus, the invention provides methods for identifying homing molecules that bind to angiogenic vasculature in non-tumor tissue as well as homing molecules that home to angiogenic vasculature of a tumor. As disclosed herein, a substantially

purified NGR receptor can be used to identify homing molecules that home to non-tumor neovascularized tissues of a subject, as well as to identify tumor homing molecules.

A homing molecule that homes to angiogenic vasculature or a tumor homing molecule that homes to angiogenic vasculature of a tumor is identified by screening one or more molecules, for example, a library of molecules. As used herein, the term "library" means a collection of molecules. A library can contain a few or a large number of different molecules, varying from about ten molecules to several billion molecules or more. If desired, a molecule can be linked to a tag, which can facilitate recovery or identification of the molecule. As disclosed herein, a homing molecule that homes to angiogenic vasculature can be identified by in vitro screening against a substantially purified NGR receptor.

As used herein, the term "molecule" is used broadly to mean an organic chemical such as a drug; a nucleic acid molecule such as an RNA, a cDNA or an oligonucleotide; a peptide, including a variant or modified peptide or peptide-like molecules, referred to herein as peptidomimetics, which mimic the activity of a peptide; or a protein such as an antibody or a growth factor receptor or a fragment thereof such as an Fv, single chain Fv(scFv), Fd or Fab fragment of an antibody, which contains a binding domain. For convenience, the term "peptide" is used broadly herein to mean peptides, proteins, fragments of proteins and the like, which can have, for example, a cyclic or linear conformation. A molecule also can be a non-naturally occurring molecule, which does not occur in nature, but is produced as a result of in vitro methods, or can be a naturally occurring molecule such as a protein or fragment thereof expressed from a cDNA library or a peptidomimetic.

A molecule to be screened against a substantially purified NGR receptor according to a method of the invention can be a "peptidomimetic," which is used broadly to mean a peptide-like molecule that has the binding activity of a tumor homing peptide, such as a peptidomimetic analog of an NGR peptide. Thus, peptidomimetics, including chemically modified peptides, peptide-like molecules containing non-naturally occurring amino acids, peptoids and the like, and, in particular, peptidomimetics of an NGR containing peptide, can be screened for the ability to specifically bind an NGR receptor, and thus, for activity in homing to angiogenic vasculature (see, for example, "Burger's Medicinal Chemistry and Drug Discovery" 5th ed., vols. 1 to 3 (ed. M. E. Wolff; Wiley Interscience 1995), which is incorporated herein by reference). Peptidomimetics provide various advantages over a peptide, for example, increased stability during passage through the digestive tract and, therefore, are advantageously used for oral administration.

Collections or libraries of peptidomimetics are well known in the art, for example, databases that contain libraries of potential peptidomimetics. For example, the Cambridge Structural Database contains a collection of greater than 300,000 compounds that have known crystal structures (Allen et al., *Acta Crystallogr.* Section B, 35:2331 (1979)). This structural depository is continually updated as new crystal structures are determined and can be screened for compounds having suitable shapes, for example, the same shape as a tumor homing molecule such as an NGR peptide, as well as potential geometrical and chemical complementarity to a target molecule bound by a tumor homing peptide. Where no crystal structure of a tumor homing peptide or a target molecule, which binds the tumor homing molecule, is available, a structure can be generated using, for example, the program CONCORD (Rusinko et al., *J. Chem. Inf. Comput. Sci.* 29:251 (1989)). Another database, the Available Chemicals Directory (Molecular Design Limited, Informations Systems; San Leandro Calif.), contains about 100, 000 compounds that are commercially available and can be screened to identify a tumor homing molecule or a homing molecule that homes to angiogenic vasculature according to a method of the invention.

Methods for preparing libraries containing diverse populations of various types of molecules such as peptides, peptoids and peptidomimetics are well known in the art and various libraries are commercially available (see, for example, Ecker and Crooke, *Biotechnology* 13:351–360 (1995), and Blondelle et al., *Trends Anal. Chem.* 14:83–92 (1995), and the references cited therein, each of which is incorporated herein by reference; see, also, Goodman and Ro, *Peptidomimetics for Drug Design*, in "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1 (ed. M. E. Wolff; John Wiley & Sons 1995), pages 803–861, and Gordon et al., *J. Med. Chem.* 37:1385–1401 (1994), each of which is incorporated herein by reference). Where a molecule is a peptide, protein or fragment thereof, the molecule can be produced in vitro directly or can be expressed from a nucleic acid, which can be produced in vitro. Methods of synthetic peptide and nucleic acid chemistry are well known in the art.

A library of molecules also can be produced, for example, by constructing a cDNA expression library from mRNA collected from a cell, tissue, organ or organism of interest. Methods for producing such libraries are well known in the art (see, for example, Sambrook et al., *Molecular Cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press 1989), which is incorporated herein by reference). Preferably, a peptide encoded by the cDNA is expressed on the surface of a cell or a virus containing the cDNA. For example, cDNA can be cloned into a phage vector such as fuse 5 (Example I), wherein, upon expression, the encoded peptide is expressed as a fusion protein on the surface of the phage.

In addition, a library of molecules can comprise a library of nucleic acid molecules, which can be DNA or RNA or an analog thereof. Nucleic acid molecules that bind, for example, to a cell surface receptor are well known (see, for example, O'Connell et al., *Proc. Natl. Acad. Sci., USA* 93:5883–5887 (1996); Tuerk and Gold, *Science* 249:505–510 (1990); Gold et al., *Ann. Rev. Biochem.* 64:763–797 (1995), each of which is incorporated herein by reference). Thus, a library of nucleic acid molecules can be contacted with a substantially purified NGR receptor to identify a tumor homing molecule or a homing molecule that homes to angiogenic vasculature. If desired, the nucleic acid molecules can be nucleic acid analogs that, for example, are less susceptible to attack by nucleases (see, for example, Jelinek et al., *Biochemistry* 34:11363–11372 (1995); Latham et al., *Nucl. Acids Res.* 22:2817–2822 (1994); Tam et al., *Nucl. Acids Res.* 22:977–986 (1994); Reed et al., *Cancer Res.* 59:6565–6570 (1990), each of which is incorporated herein by reference).

Particularly useful libraries of molecules to be screened for specific binding to an NGR receptor and, therefore, for activity in homing to angiogenic vasculature, include phage display libraries. Such phage display libraries of molecules include secondary libraries expressing NGR in various contexts, including cyclic phage display peptide libraries such as X_2CNGRCX_2 (SEQ ID NO:222), CX$_2$(C/X)NGR(C/X)X$_2$C (SEQ ID NO:223), and CNGRCX$_6$ (SEQ ID NO:224) (where "C" is cysteine and "X" is any amino acid; see Example X). A library of molecules to be screened also can be a library of antibodies or antibody fragments such as Fv, single chain Fv or Fab fragments; as disclosed in

Example X, such a library can be, for example, a combinatorial scfv library prepared from rabbits immunized with human tumor xenografts. Such antibodies can bind to the same epitope recognized by the anti-CD13 antibodies F23 and MY7, or can bind to a different epitope.

One skilled in the art understands that a molecule that specifically binds a substantially purified NGR receptor can bind and modulate the activity of the NGR receptor, or can be inert with respect to its ability to affect the activity of an NGR receptor. As disclosed herein, for example, an NGR receptor is functionally important in angiogenesis. A molecule that specifically binds a substantially purified NGR receptor can be an agonist or an inhibitor of the receptor and, thus, can enhance or inhibit angiogenesis.

An inhibitor of an NGR receptor can be highly specific for the NGR receptor. For example, a specific inhibitor can be an antibody that binds with high specificity to an NGR receptor. The antibody can have the inherent property of inhibiting NGR receptor activity upon binding of the antibody. Alternatively, an antibody that is not inhibitory but binds specifically to an NGR receptor can be conjugated to a drug or target to generate a specific inhibitor. The antibody can be a monoclonal or polyclonal antibody or can be a functional antibody fragment such as a Fv, single chain Fv or Fab fragment.

Accordingly, monoclonal or polyclonal antibodies exhibiting specific binding to an NGR receptor can be generated by methods well known to those skilled in the art (Harlow and Lane, *Antibodies: A laboratory manual* Cold Spring Harbor Laboratory Press 1988), which is incorporated herein by reference). Alternatively, libraries of functional antibody fragments, which can bind to an NGR receptor, can also be screened to identify a homing molecule that binds to an NGR receptor. For example, a combinatorial scfv library generated by immunizing with human tumor xenografts or a substantially purified NGR receptor can be screened for binding to an NGR receptor (see Example X).

In addition to inhibitors that are highly specific for an NGR receptor, inhibitors also can exhibit inhibitory activity to other molecules related but not identical to an NGR receptor. For example, aminopeptidase inhibitors can exhibit activity specific for an aminopeptidase or can exhibit inhibitory activity to several aminopeptidases. A homing molecule that binds to an NGR receptor and is an aminopeptidase inhibitor is particularly useful if the inhibitor exhibits preferential binding to an NGR receptor in a target neovascularized tissue.

Accordingly, libraries of molecules to be screened for activity in specifically binding a substantially purified NGR receptor include structural analogs of natural substrates of aminopeptidase as well as structural analogs of aminopeptidase inhibitors. Such libraries can include structural analogs of substrates such as Ala-PNA; Leu enkephalin; Met enkephalin or tuftsin (Xu et al., *Experimental Hematology* 25:521–529 (1997), which is incorporated herein by reference). Such libraries also can include structural analogs of aminopeptidase inhibitors, such as actinonin; amastatin; bestatin; 1,10-phenanthroline or o-phenanthroline; or 2,2'-dipyridyl; or analogs of Phe-Leu (Xu et al., supra, 1997). Such libraries can additionally include structural analogs of fumagillin (Sin et al., *Proc. Natl. Acad. Sci. USA* 94:6099–6103 (1997), which is incorporated herein by reference). From the above, it is understood that a molecule that specifically binds a substantially purified NGR receptor can be a naturally or non-naturally occurring structural analog of Phe-Leu.

In addition to screening phage and DNA libraries as described above, combinatorial chemistry libraries also can be screened in vitro using a substantially purified NGP receptor according to a method of the invention. Methods for generating combinatorial libraries are well known in the art as described, for example, in Gordon et al., *J. Med. Chem.* 37:1385–1401 (1994); Gallop et al., *J. Med. Chem.* 37:1203–1251 (1994); and Wilson and Czarnik, eds., *Combinatorial Chemistry* John Wiley & Sons, New York (1997), each of which is incorporated herein by reference).

The presence of a tumor homing molecule or a homing molecule that specifically binds an NGR receptor within a library of molecules can be identified using various methods well known in the art. Generally, the compounds in a library can be tested individually, for example, using high throughput screening. If desired, the individual compounds can be tagged to facilitate recovery or identification of the molecule. Such tagged libraries are useful for in vivo and in vitro screening.

As used herein, the term "tag" means a physical, chemical or biological moiety such as a plastic microbead, an oligonucleotide or a bacteriophage, respectively, that is linked to a molecule of the library. Methods for tagging a molecule are well known in the art (Hermanson, *Bioconlugate Techniques* (Academic Press 1996), which is incorporated herein by reference).

A specific tag can be particularly useful in the methods of the invention for identifying a tumor homing molecule or a homing molecule that homes to angiogenic vasculature. As used herein, the term "specific tag" means a physical, chemical or biological tag that is linked to a particular molecule in a library and is unique for that particular molecule. A specific tag is particularly useful if it is readily identifiable. A nucleotide sequence that is unique for a particular molecule of a library is an example of a specific tag. For example, the method of synthesizing peptides tagged with a unique nucleotide sequence provides a library of molecules, each containing a specific tag, such that upon determining the nucleotide sequence, the identity of the peptide is known (see Brenner and Lerner, *Proc. Natl. Acad. Sci., USA* 89:5381–5383 (1992), which is incorporated herein by reference). The use of a nucleotide sequence as a specific tag for a peptide or other type of molecule provides a simple means to identify the presence of the molecule in a sample because an extremely sensitive method such as PCR can be used to determine the nucleotide sequence of the specific tag, thereby identifying the sequence of the molecule linked thereto. Similarly, the nucleic acid sequence encoding a peptide expressed on a phage is another example of a specific tag, since sequencing of the specific tag identifies the amino acid sequence of the expressed peptide.

Identified tumor homing molecules are useful, for example, for targeting a desired moiety such as a drug, a toxin or a detectable label, which can be linked to the molecule, to a tumor. In addition, a tumor homing molecule is useful for identifying the target molecule, to which the homing molecule binds in the tumor. Once a target molecule has been identified, for example, an NGR receptor as disclosed herein, the target molecule can be used to identify additional tumor homing molecules.

The present invention also provides a method of directing a moiety to angiogenic vasculature of a tumor in a subject by administering to the subject a conjugate including a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the conjugate is directed to angiogenic vasculature of a tumor. In a method

of the invention, the tumor homing molecule can be, for example, a peptide containing the sequence NGR, and, if desired, can be part of a conjugate in which the moiety is a cytotoxic agent, drug or chemotherapeutic agent, for example, doxorubicin. A tumor homing peptide containing the sequence NGR can have, for example, the sequence CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or CNGRC (SEQ ID NO:8). In a method of the invention for directing a moiety to angiogenic vasculature of a tumor in a subject, the tumor homing molecule also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin and can be linked, if desired, to a doxorubicin moiety.

The present invention additionally provides a method of inhibiting angiogenesis in a tumor of a subject by administering to the subject a conjugate including a moiety linked to a tumor homing molecule that exhibits specific binding to an NGR receptor, whereby the conjugate is directed to angiogenic vasculature of a tumor. In a method of the invention, the tumor homing molecule can be, for example, a peptide containing the sequence NGR, and, if desired, can be part of a conjugate in which the moiety is a cytotoxic agent, drug or chemotherapeutic agent, for example, doxorubicin. A tumor homing peptide containing the sequence NGR can have, for example, the sequence CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7) or CNGRC (SEQ ID NO:8). In a method of the invention for directing a moiety to angiogenic vasculature of a tumor in a subject, the tumor homing molecule also can be, for example, an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin and can be linked, if desired, to a doxorubicin moiety.

The invention also provides a method of inhibiting angiogenesis in a non-tumor tissue. The method includes administering a conjugate including a moiety linked to a homing molecule that exhibits specific binding to an NGR receptor, whereby the conjugate is directed to angiogenic vasculature of a non-tumor tissue. Inhibiting angiogenesis in a non-tumor tissue is useful, for example, for treating diseases involving neovascularized tissue such as retinal neovascularization in macular degeneration and diabetes and neovascularization in rheumatoid arthritis synovium.

As disclosed, tumor homing molecules can be conjugated to moieties such as a drug or toxin in order to target the drug or toxin to a tumor. A tumor homing molecule such as one of the NGR containing peptides CNGRCVSGCAGRC (SEQ ID NO:3), NGRAHA (SEQ ID NO:6), CVLNGRMEC (SEQ ID NO:7), or CNGRC (SEQ ID NO:8) can be used to direct a moiety to angiogenic vasculature. Additional tumor homing molecules that bind to the NCGR receptor identified in vivo or in vitro as described above also can be used to direct a moiety to the angiogenic vasculature of a tumor.

In addition to tumor homing molecules that. contain NGR, other tumor homing molecules that specifically bind to the NGR receptor are also useful for targeting angiogenic vasculature. As described below, the NGR receptor exhibits aminopeptidase activity, and the aminopeptidase activity can be inhibited by known inhibitors such as bestatin, o-phenanthroline and actinonin. Therefore, in addition to NGR containing peptides, other molecules that bind to the NGR receptor can also function as tumor homing molecules. For example, a molecule that functions as an aminopeptidase inhibitor such as bestatin, o-phenanthroline, actinonin, amastatin, 2,2'-dipyridyl or fumagillin can be used as conjugates with a drug or toxin to home to angiogenic vasculature.

The invention additionally provides a method of directing a moiety to angiogenic vasculature in a subject by administering to the subject a conjugate comprising a moiety linked to a homing molecule that exhibits specific binding to an NGR receptor, where the moiety is directed to angiogenic vasculature. Thus, the invention provides a method of directing a conjugate, for example, of a drug or toxin, to angiogenic vasculature of non-tumor tissue. The targeting of a conjugate to angiogenic vasculature of non-tumor tissue is useful, for example, for treating diseases involving neovascularized tissue such as retinal neovascularization in macular degeneration and diabetes and neovascularization in rheumatoid arthritis synovium.

A variety of moieties can be directed to angiogenic vasculature in a method of the invention. As used herein, the term "moiety" is used broadly to mean a physical, chemical, or biological material that is linked to a tumor homing molecule for the purpose of being targeted in vivo to a tumor or to angiogenic vasculature expressing a target molecule recognized by the tumor homing molecule. In particular, a moiety is a biologically useful moiety such as therapeutic moiety, a diagnostic moiety or a drug delivery vehicle. Thus, a moiety can be a therapeutic agent, for example, a cancer chemotherapeutic agent such as doxorubicin, which, when linked to a tumor homing molecule, provides a conjugate useful for treating a cancer in a subject. In addition, a moiety can be a drug delivery vehicle such as a chambered microdevice, a cell, a liposome or a virus, which can contain an agent such as a drug or a nucleic acid.

A moiety also can be a molecule such as a polypeptide or nucleic acid, to which a tumor homing molecule is grafted for the purpose of directing the polypeptide or nucleic acid to a selected tumor (Smith et al., *J. Biol. Chem.* 269:32788–32795 (1994); Goldman et al., *Cancer Res.* 15:1447–1451 (1997), each of which is incorporated herein by reference). For example, a peptide tumor homing molecule can be expressed as a fusion protein with a desired polypeptide such that the peptide targets the grafted polypeptide to a selected tumor. Such a desired polypeptide, which is grafted to the tumor homing peptide, can be a polypeptide involved in initiating a cell death pathway, for example, caspase 8, thus providing a means to direct caspase 8 to a tumor, where it can induce apoptosis of the tumor cells or of the vasculature supplying the tumor. A tumor homing peptide also can be grafted to a polypeptide expressed by a virus, for example, the adenovirus penton base coat protein, thus providing a means to target a virus to a tumor (Wickham et al., *Gene Ther.* 2:750–756 (1995); Weitzman et al., In: "Gene Therapy and Vector Systems" 2:17–25 (1997), each of which is incorporated herein by reference; see, also, Example III). Such a grafted virus can contain an exogenous gene useful in a method of gene therapy. Accordingly, the invention provides compositions of matter comprising a tumor homing molecule/moiety conjugate.

A moiety can be a detectable label such a radiolabel or can be a cytotoxic agent, including a toxin such as ricin or a drug such as a chemotherapeutic agent or can be a physical, chemical or biological material such as a liposome, microcapsule, micropump or other chambered microdevice, which can be used, for example, as a drug delivery system. Generally, such microdevices, should be nontoxic and, if desired, biodegradable. Various moieties, including microcapsules, which can contain an agent, and methods for linking a moiety, including a chambered microdevice, to a molecule of the invention are well known in the art and commercially available (see, for example, "Remington's Pharmaceutical Sciences" 18th ed. (Mack Publishing Co.